

DESIGN AND USE OF ADVANCED ZINC CHELATING PEPTIDES TO REGULATE MATRIX METALLOPROTEINASES

5 FIELD OF THE INVENTION

The present invention relates to compositions and methods for enhancing wound healing, especially chronic wounds (e.g., diabetic wounds, pressure sores). More specifically, the invention relates to improved wound healing through regulation of matrix metalloproteinase
10 activity.

BACKGROUND OF THE INVENTION

In normal tissues, cellular connective tissue synthesis is offset by extracellular matrix degradation, the two opposing effects
15 existing in dynamic equilibrium. Degradation of the matrix is brought about by the action of matrix metalloproteinases (MMPs) released from resident connective tissue cells and invading inflammatory cells. Normally, these catabolic enzymes are tightly regulated at the level of their synthesis and secretion and also at the level of their extracellular
20 activity. Extracellular control occurs primarily by regulation with specific enzymes, such as TIMPs (tissue inhibitors of metalloproteinases), which form complexes with MMPs. These complexes prevent MMP action. Cellular level control of MMP activity occurs primarily by regulating MMP gene expression and y down

regulating the expression of the membrane bound MMPs (MT-MMP) that activate the excreted proenzyme form of the MMP.

MMPs are a family of neutral metalloenzymes capable of degrading extracellular matrix (ECM) macromolecules. Members of this family that have been isolated and characterized include interstitial fibroblast collagenase, stromelysin, and type IV collagenase. Other potential members include a poorly characterized 94,000 dalton gelatinase and several low molecular weight gelatinases and telopeptidases. Structurally, MMPs contain a zinc(II) ionic site at the active site of the protein. Binding of zinc to the ionic site is required for hydrolytic activity.

TIMPs are glycoproteins that specifically regulate interstitial collagenase on a 1:1 stoichiometric basis. That is, TIMPs form very specific regulatory complexes with MMPs, only regulating a specific subset of the MMPs. No naturally occurring TIMP molecule singly regulates all types of MMPs.

In chronic wounds, the ratio of MMPs to TIMPs is high, such that most of the MMPs are unregulated. This unregulated MMP activity results in the accelerated, uncontrolled breakdown of the ECM, leading to destruction of the newly formed wound bed. Additionally, the concomitant elevation of proteinase levels, cause hydrolyzation of TIMP molecules, further increasing the MMP to TIMP ratio.

multiple sclerosis; and angiogenesis dependent diseases, which include
angiofibromas, hemangioma, solid tumors, blood-borne tumors,
leukemia, metastasis, telangiectasia, psoriasis, scleroderma, pyogenic
granuloma, myocardial angiogenesis, plaque neovascularization,
5 coronary collaterals, ischemic limb angiogenesis, corneal diseases,
rubeosis, neovascular glaucoma, diabetic retinopathy, retrolental
fibroplasia, arthritis, diabetic neovascularization, macular degeneration,
wound healing, peptic ulcer, fractures, keloids, vasculogenesis,
hematopoiesis, ovulation, menstruation, and placentation.

10 Given the large number of diseases associated with MMP
activity, there is a need to control MMP activity. Several approaches
have been suggested to accomplish such regulation. One approach has
focused on the catalytic role of zinc in MMPs, designing zinc-chelating
regulators. Potent regulators have been generated by introducing zinc
15 chelating groups, such as peptide hydroxamates and thiol-containing
peptides, into substrates. Peptide hydroxamates and TIMPs have been
successfully used in animal models to treat cancer and inflammation.
While these hydroxamates are potent at regulators of MMPs by binding
to zinc, they are toxic to humans because they bind to all zinc-containing
20 enzymes. Because many biochemical reactions occurring in the body
require zinc, use of the hydroxamates detrimentally effects these other
functions and can result in death.

created by partly modifying the amino acid sequence of natural elafin. The modification can be created by either chemical synthesis or site-directed mutagenesis.

U.S. Patent No. 5,464,822 to Christophers *et al.* discloses a
5 polypeptide that possesses inhibitory activity against human leukocyte elastase. The polypeptides possess inhibitory activity that is specific for serine proteases. For example, they possess inhibitory activity against proteases, such as human leukocyte elastase and porcine pancreatic elastase, but do not possess any significant inhibitory activity against
10 trypsin. These polypeptides can be prepared by genetic engineering or obtained from psoriatic scales of human skin.

U.S. Patent No. 5,698,671 to Stetler-Stevenson *et al.* discloses a protein defined by the presence of specific cysteine-containing amino acid sequences, isolated from the conditioned media
15 of cultured human tumor cells, that binds with high affinity to MMPs and analogs thereof. The particular inhibitor is made by preparing peptides and proteins having a cysteine residue at the same interval as that of the various tissue inhibitors of metalloproteinase (TIMPs). The peptides must have at least two appropriately spaced cysteines to ensure
20 inhibitory activity by virtue of a disulfide bridge formation. In addition, the invention discloses a method for purifying natural MMP inhibitors by MMP affinity chromatography.

Despite these varied approaches, the current art does not selectively regulate MMP activity. Traditionally, high affinity regulators have been utilized, resulting in complete MMP inhibition. However, shutting off all MMP activity is actually deleterious to the healing process, as some MMP activity is required for tissue remodelling. For example, potent inhibition aimed at binding the zinc (II) site is toxic to humans because it shuts off bind to all zinc-containing enzymes. It is therefore necessary to have regulation be selective.

Thus, there is a need in the art for improved regulation of MMPs to promote healing of chronic and acute wounds.

There is also a need in the art for an inhibitor having relatively good affinity, which is selective.

Furthermore, there is a need in the art for MMP inhibitors that are not toxic to the individual to whom they are administered.

SUMMARY OF THE INVENTION

Using a novel approach to wound site proteinase management, the present invention, in one aspect, provides a new class of MMP regulators. These regulators comprise a zinc chelator covalently attached to a peptide that corresponds to a region of a TIMP protein that directly interacts with an MMP molecule in the vicinity of the catalytic zinc molecule to bind wound site proteinases. The binding

Finally, by applying these novel MMP regulators to an area to be treated, the present invention provides a method of controlling diseases cause by uncontrolled MMP activity without the use of toxic inhibitors.

Therefore, it is an object of the invention to provide new
5 MMP regulators.

It is another object of the invention to provide MMP regulators that comprises a zinc chelator and synthetic peptides containing novel amino acid sequences that bind to the TIMP binding region of the MMP.

10 It is yet another object of the invention to provide novel synthetic peptides for use in the production of the MMP regulators of the invention.

It is a further object of the present invention to provide a novel zinc chelator for use in the production of the MMP regulators of
15 the invention.

It is an object of the invention to provide synthetic peptides having SEQ ID Nos. 1, 2, and 3.

It is another object of the present invention to provide MMP regulators comprising peptides having SEQ ID Nos. 1, 2, or 3 and a zinc
20 chelator.

It is yet another object of the invention to provide methods for treating chronic and acute wounds.

It is a further object of the present invention to provide methods for treating angiogenic-associated diseases.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a time course reaction of MMP-9 hydrolysis of fluoresceinated collagen in the presence of increasing amounts of the zinc chelating peptide. The curves (from top to bottom) represent MMP-9:ChePep-1 molar stoichiometries of 1:0, 1:0.25, 1:0.5, and 1:1. ChePep-1 is composed of PSDE coupled with the peptide having SEQ ID No. 4. The graph shows fluorescence as a function of time in the continuous assay.

Figure 2 depicts ChePep-2 regulation of MMP-9 via FRET assay. ChePep-2 is composed of IDA coupled with a peptide having Seq ID No. 5. This mixture was added to the FRET substrate peptide in reaction buffer. Fluorescence measurements were made at the indicated times. The curves represent ChePep-2: MMP-9 molar stoichiometries of 0:1 (closed circles), 0.1:1 (open circles), 0.2:1 (closed squares), 0.3:1 (open squares).

Figure 3 represents ChePep-3 (closed squares) and ChePep-4 (open circles) regulation of MMP-9 via FRET assay. ChePep-

3 is made by combining PSDE with a peptide having SEQ ID No. 6 and ChePep-4 is made by combining IDA with a peptide having SEQ ID No. 6. ChePeps were incubated with MMP-9 for 10 minutes to effect binding. This mixture was added to the FRET substrate peptide in reaction buffer. Fluorescence measurements were made at the indicated times. MMP-9 only control is shown for reference (closed circles). ChePep:MMP-9 molar stoichiometry is 0.25:1.

Figure 4 illustrates ChePep-5 regulation of MMP-9 via FRET assay. ChePep-5 is composed of a AFTA combined with a peptide having SEQ ID No. 7. ChePep-5 was incubated with MMP-9 for 10 minutes to effect binding. This mixture was added to the FRET substrate peptide in reaction buffer. Fluorescence measurements were made at the indicated times. The curves represent ChepPep-5: MMP-9 molar stoichiometries of 0:1 (closed circles), 0.1:1 (open circles), 0.2:1 (closed squares), 0.3:1 (open squares). Excitation was at 365 nm, and the emission was fixed at 450 nm.

Figure 5 shows ChePep-6 regulation of MMP-9 via the FRET assay. ChePep-6 is made by combining AFTA with a peptide having SEQ ID No. 8. ChePep-6 was incubated with MMP-9 for 10 minutes to effect binding. This mixture was added to the FRET substrate peptide in reaction buffer. Fluorescence measurements (Excitation 365 nm, Emission 450 nm) were made at the indicated times.

The curves represent ChepPep-6: MMP-9 molar stoichiometries of 0:1 (closed circles), 0.1:1 (open circles).

Figure 6 depicts SPR analysis of ChePep-6 binding to immobilized MMP-9 that is on the surface of a BiaCoreCM-5 chip. The sensogram shows the relative SPR signal as a function of time for a 400 second association phase and an 800 second dissociation phase. Flow rate was maintained at a rate of 20 μ L per minute.

Figure 7 represents compound viability assays. The graph plots the percent viability of the peptides utilized in this study relative to a PBS control. Error bars are +/-SD. Samples (left to right) are as follows: PBS positive control, 1%Triton X-100 negative control, ChePep-1, ChePep-2, ChePep-3, ChePep-4, ChePep-5, and ChePep-6.

Figure 8 is a reaction scheme for modification of EDTA to PSDE to allow coupling of the EDTA chelator to the peptides of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

MMPs contain a zinc molecule located in the active site. This zinc molecule intimately participates in the chemistry of degrading collagen. As a result, if the zinc is blocked or removed, enzymatic activity of the MMP can be inhibited. The present invention provides compositions (referred to herein as ChePeps) for the regulation of MMP

acids in length. The peptide having SEQ ID No. 3 is 9 amino acids in length encompasses MMP residues 97-105.

Within these preferred peptides, there is a large degree of sequence variation, as denoted by positions in parentheses. This variability can be used as a tool to modulate binding affinity and specificity of TIMP binding interactions. The exact sequence of the peptide can be altered to modulate binding affinity and/or specificity, so long as there is a single cysteine residue in the peptide. These three preferred peptides can be further modified by C-ter amidation and N-ter acetylation. This charge neutralization is often found to increase binding affinity as it mimics the peptide being in the context of a whole protein. In sum, these specific peptides allow for altering the level of specific MMPs in a coordinated manner.

Table 1. Peptide Family Sequences

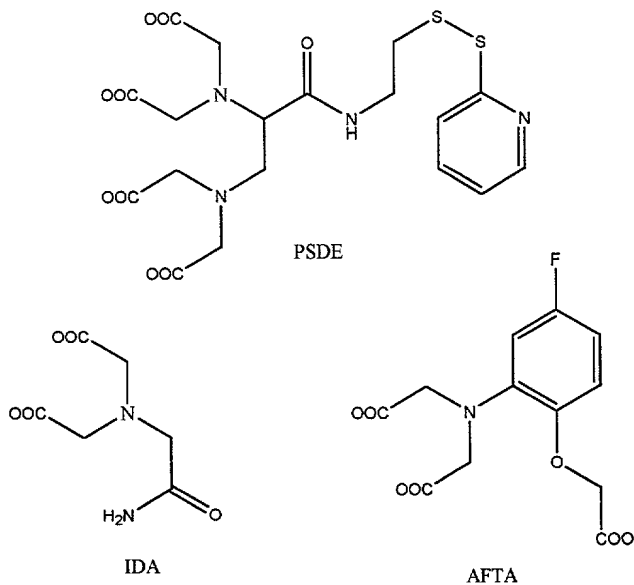
SEQ ID No. 1	C-(S/T)-C-(S/A/V)-P-H-P
SEQ ID No. 2	(I/V)-(E/Q/R)-(F/Y)-(I/V)-(Y/H)-T-(A/P/E)-(P/F/A)- (S/D/M)-(A/S)-(V/L)-(C/G)
SEQ ID No. 3	(M/V/L)-(H/F/Y)-(I/T)-(T/H/G)-(L/T)-C-(D/N/S)- (F/Y)-(I/V)

While not wishing to be bound by the following theory, it appears that the preferred ChePep complexes block the imino carboxylate groups via zinc chelation prior to activating the remaining carboxyl group for coupling to the peptides. Once liganded to the zinc

peptides can be coupled to the targeting peptide via standard peptide synthesis.

In a preferred embodiment, one of three zinc chelating molecules is covalently attached to one of the several TIMP derived peptides that comprise the present invention. The chemical structures of these three zinc chelating molecules: pyridine disulfide ethylenediaminetetraacetic acid (PSDE), amino-iminodiacetic acid (IDA), and 2-amino-4-fluorophenol *N,N,O* triacetic acid (AFTA), are depicted below in Structure 1.

Structure 1



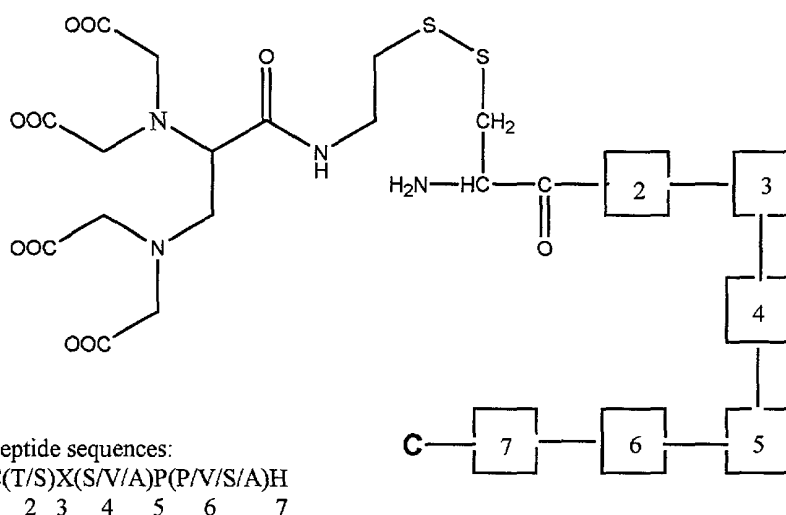
Pyridine disulfide ethylenediaminetetraacetic acid (PSDE) of the present invention is produced in a literature synthesis. (Hayward

et al (1995), J. Org. Chem. 60, 3924-27) Covalent coupling of PDSE to the peptide is carried out by a simple stoichiometric disulfide exchange with a cysteine at the amino terminus. Typically the process results in a substantially pure product with an overall yield of about 10-25 percent.

5 The peptide sequence shown in Structure 2 below is a modification of SEQ ID No. 1 wherein the cysteine in position 3 is replaced with an alanine. This molecule can be directly coupled to any free thiol via a simple disulfide exchange reaction.

10

Structure 2



In another embodiment, the present invention provides PSDE be coupled to SEQ ID No. 4 (CSAVPVH) with an overall yield of 80 percent. The reaction product is then purified via RPHPLC as described in the Example. Introduction of this molecule, ChePep-1, to

15

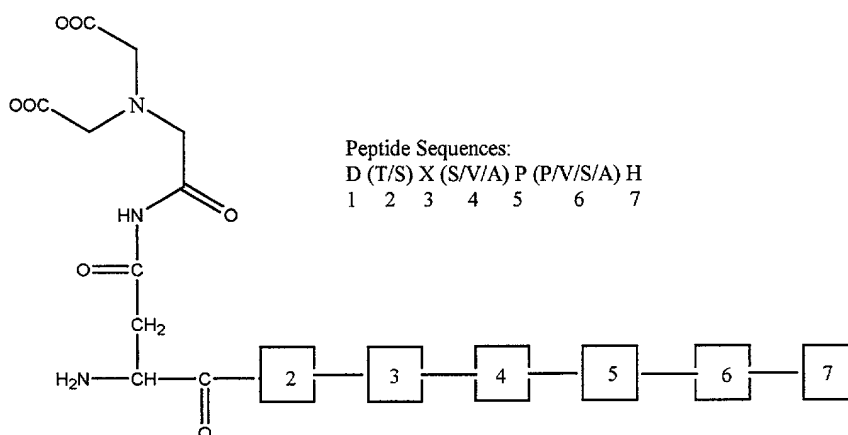
MMP-9 prevents the enzyme from degrading fluoresceinated collagen in a dose dependent manner as is shown in Figure 1.

In yet another embodiment, the present invention provides creation of a combined regulator (binding specificity via the peptide and
5 zinc chelation via the small molecule) by covalently joining EDTA or IDA to a TIMP derived peptide sequence. Ethylenediaminetetraacetic acid (EDTA) and iminodiacetic acid (IDA) are potent chelators of zinc. However, an EDTA chelator needs some prior literature synthesis in order to perform a coupling reaction preferred by the present invention.
10 This synthesis is described by Hayward *et al.* (J. Org. Chem., Vol 60(12), 1995, 3924-3927) and is presented in Figure 8. Such synthesis is not required for IDA because it is relatively easy to couple IDA to the carboxylate group of an aspartate that has been converted into a succinimide ester upon reaction with N-hydroxysuccinimide (NHS)/ N-ethyl-N'-(dimethylaminopropyl)carbo-diimide (EDC) (EDC/NHS).
15

By coupling IDA to a similar peptide, SEQ ID No. 5 (DSAVPVH), the effect of the size of the EDTA moiety on ChePep-1 (PSDE and SEQ ID No.4) can be determined. In this instance, the carboxylate group of the N-ter peptide aspartic acid is activated to a
20 succinimide ester via incubation with EDC/NHS. This straightforward reaction is commonly used to link free amino groups with activated carboxylates. The general structure of a peptide that is covalently

coupled to IDA through an activated aspartic acid carboxylate is shown below in Structure 3.

Structure 3



5

Preferably, the entire synthetic scheme that couples IDA to the peptide and purifies the final product proceeds with an overall 49 percent yield. To avoid possible contaminants in the reaction, use a peptide with a C-ter amide group. Hence the only free carboxyl group in the peptide should be the aspartic acid sidechain.

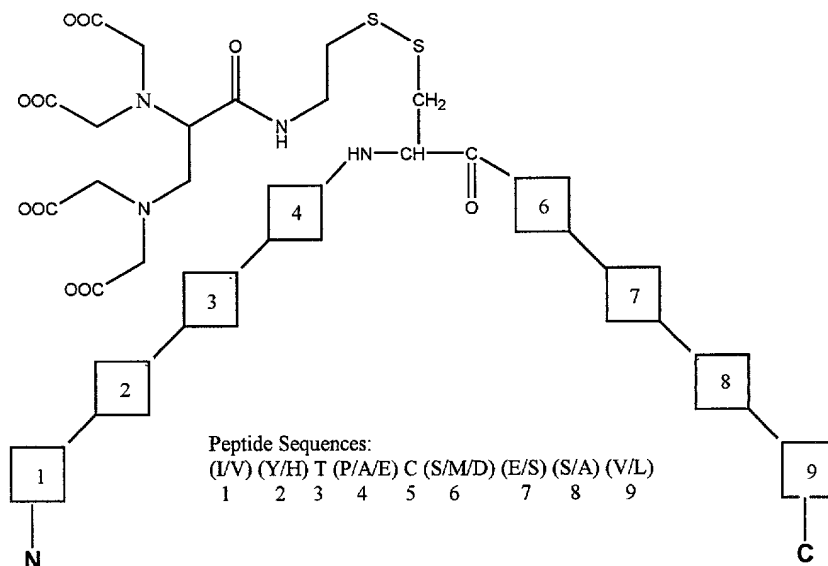
The molecule ChePep-2 (IDA and SEQ ID No. 5) regulates MMP-9 in the fluorescence resonance energy transfer (FRET) assay in a dose dependent manner. The construct is significantly more effective in regulating MMP-9 than was ChepPep-1, as demonstrated in Figure 2.

This may be due to the ability of an IDA moiety to more effectively ligate the active site zinc than PSDE because of steric considerations as

well as the need to satisfy charge interactions with the second chelating group on PSDE. In addition, the neutralization of the C-ter negative charge on the peptide via the amidation may also alter binding affinity.

In another embodiment, PSDE is coupled to a peptide of SEQ ID No. 2. Specifically, PSDE is coupled to a peptide having SEQ ID No. 10 that is aminated at the amino terminus (IYTACMSAV-NH₂) via the same disulfide exchange reaction previously discussed. The yield for the final product was 37%. The generalized structure of a chelating peptide (ChePep) composed of PSDE and SEQ ID No. 2 is shown below.

Structure 4



If the cysteine residue in the fifth position of the above molecule is altered to aspartic acid, then IDA can be incorporated into the structure at the same position. In a preferred embodiment, IDA is coupled to SEQ ID No. 2, specifically, the peptide of SEQ ID No. 11 via
 5 the EDC/NHS reaction described in the Examples section. The final product yield was 75 percent. RP-HPLC chromatograms of both ChePep molecules revealed a single 214 nm absorbing peak.

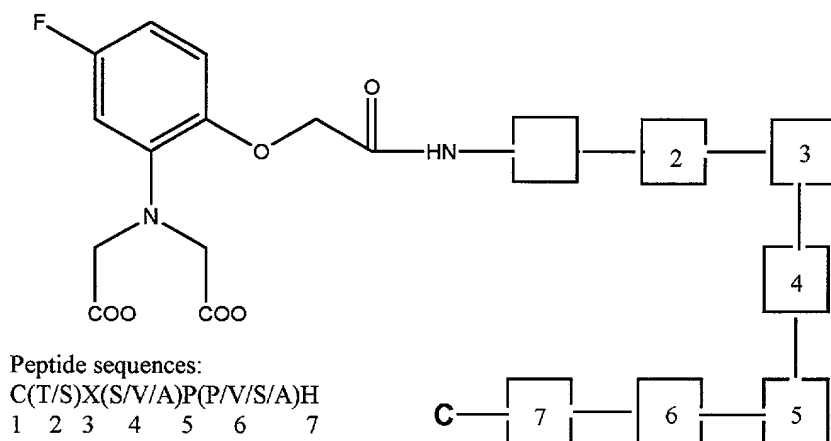
Both ChePep-3 (PSDE and SEQ ID No. 6) and ChePep-4 (IDA and SEQ ID No. 6) show nearly identical ability to regulate MMP-
 10 9 in the standard FRET assay. The time dependent regulation course is displayed in Figure 3. Despite the fact that there are differences between IDA and PSDE, molecular modeling indicates that the chelating groups of both ChePep constructs have similar accessibility to the MMP active site zinc. In fact, the second chelating group of PSDE does not make
 15 any energetically unfavorable interactions that would lower binding affinity. It is believed that ChePep-3 may gain some binding energy by the interaction of the carboxylate oxygen on the free liganding moiety with a MMP-9 backbone amide.

Molecules like 2-amino-4-fluorophenol *N,N,O* triacetic acid
 20 (AFTA) are not, according to scientific literature, generally known or used as metal chelators, but can in fact readily chelate zinc in this application. AFTA is found to bind well into the active site of MMP-like enzymes. The diacetate group off the 2 ring position chelates the

zinc. The third acetate group can be used to couple a peptide to the molecule. The electronegative fluorine may also contribute to MMP regulation by making specific interactions in the enzyme active site. The general structure of SEQ ID No. 3 covalently linked to AFTA is depicted

5 below.

Structure 5



The above structure is created by coupling a peptide having
 10 SEQ ID No. 7 (VHTHLCD) to an AFTA zinc chelator using the same
 EDC/NHS chemistry previously described, except that the activated
 carboxyl group was donated by the AFTA moiety. Since there are three
 free carboxyl groups found on AFTA, only two of which can participate
 in zinc ligation (see Structure 1), it is necessary to make those chelating
 15 groups unreactive to EDC/NHS treatment. This can be accomplished by
 mixing AFTA with zinc sulfate prior to EDC/NHS activation. The zinc
 ion is chelated by the iminodiacetic acid moiety, leaving a single free

carboxylate. The succinimide ester is then formed on this group as described in the Examples section. The activated AFTA-zinc complex is coupled to the N-ter amino group of the peptide, and the resulting molecule (ChePep-5) is purified to homogeneity by RP-Phase HPLC.

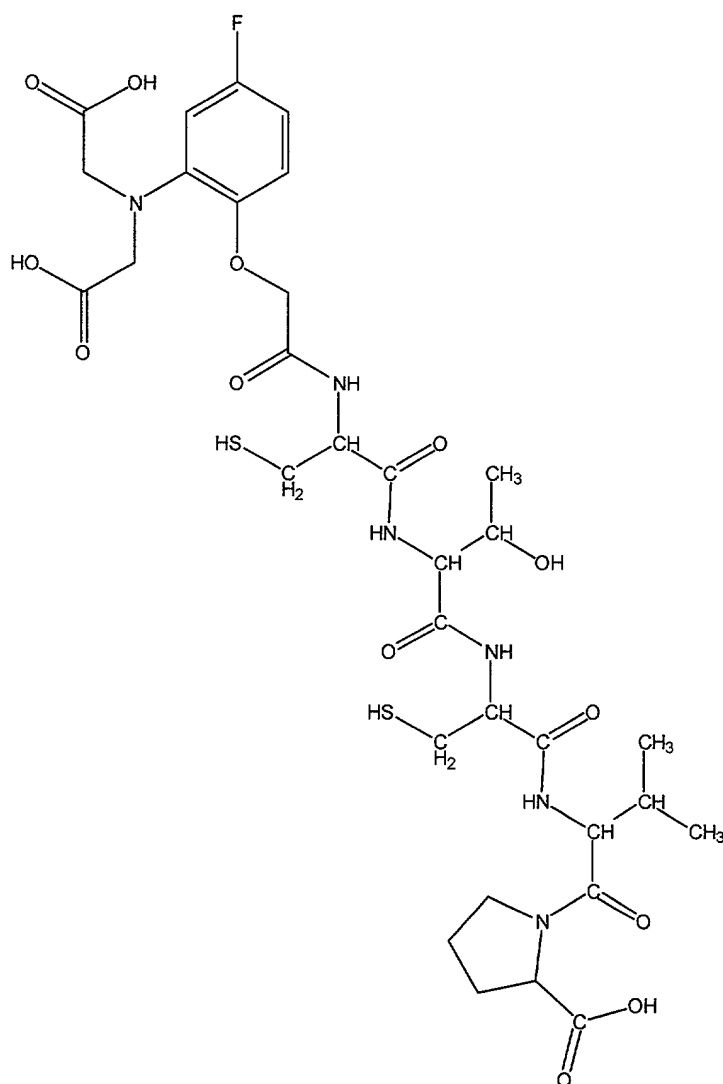
- 5 Preferably, the total recovery yield was 61% and the final material is judged to be 97% pure. The generalized structure of this type of regulator, shown in Structure 5, is capable of preventing the hydrolysis of the FRET peptide in a dose dependent manner.

- In fact, according to the molar stoichiometries test depicted in Figure 4, ChePep-5 proves to be a better regulator than the IDA or the PSDE based chelating peptides. Its success can be attributed to, but not limited to, four factors: the fluorine group which, because of its electron negativity, tenaciously binds to proteins; the ring itself, being hydrophobic, binds well to the hydrophobic active site of the MMP; the presence of three carboxyl groups to bind to allow for zinc chelation; and the amino groups to create specificity.
- 10
15

- In another embodiment of this invention, a second AFTA peptide construct is synthesized. This peptide (ChePep-6) comprises SEQ ID No. 8 (CTCVP) and an AFTA, with the AFTA molecule coupled to the peptide amino terminus. Coupling is preferably performed by first mixing an equal amount of zinc sulfate and AFTA. The resulting complex is then activated with NHS/EDC. An amide coupling is then performed using the N-ter region of the peptide.
- 20

Alternatively, AFTA can be linked to the cysteine thiol via a disulfide exchange reaction. The structure of the preferred chelating peptide is shown in Structure 6 below.

Structure 6



5

This chelating peptide possesses the ability to prevent the hydrolysis of the FRET substrate peptide in the standard assay. As seen in Figure 5,

ChePep-6 is an effective MMP-9 regulator at low molar stoichiometries. In addition, ChePep-6 binds quickly and with high affinity; and once bound, ChePep-6 does not dissociate as seen in Figure 6. In fact, the combination of a group I peptide and an AFTA moiety resulted in the most potent regulator of all the ChePeps.

In another aspect, the present invention comprises the treatment of chronic and acute wounds. By applying the composition to the chronic wound, MMP activity can be regulated. Specifically, the composition comprises a peptide with binding specificity to bring the zinc chelator into molecular proximity of the MMP bound zinc in such a way as to allow ligation. In addition to this affinity, the exact sequence of the peptide will allow targeting of specific MMPs. This chemistry regulates the level of MMP activity to a point that promotes healing.

A highlight of this peptide based therapy is the plasticity in which this material can be applied to a chronic wound. Where the infection is localized to the skin, a preferred formulation is an ointment, lotion or other topical formulation. The composition for topical application useful in the present invention can be made into a variety of product types. These include, but are not limited to, creams, gels, sticks, sprays, pastes, mousses, or any aqueous medium. These product types can comprise several types of carrier systems including, but not limited to, solutions, dispersions, emulsions, gels, solids, and liposomes. Additionally, the peptides can be introduced into the wound bed in a

continuous manner via delivery by the wound bandage material itself. Preferred dose ranges for treatment of chronic wounds are between approximately 0.01 and 1.0 mg/mL

Finally, another embodiment of the present invention is to
5 control specific MMP activity. Certain diseases are caused by uncontrollable MMP activity. In fact, some diseases are caused by only a certain of the nine MMP molecules. By designing peptides that correspond with a specific MMP's active site, and then combining a zinc chelator to the peptide, only targeted MMP activity is regulated with a
10 high degree of affinity and specificity.

The present invention is non-toxic in a skin equivalent model. The generally toxic effects of a non-specific zinc chelator are mitigated by the fact that the peptide portion of the ChePep construct directly targets the construct to the MMP active site in such a way as to
15 minimize chelation at secondary (non MMP) targets.

The present invention is further illustrated and supported by the following examples. However, these examples should in no way be considered to further limit the scope of the invention. To the contrary, one having ordinary skill in the art would readily understand that there
20 are other embodiments, modifications, and equivalents of the present invention without departing from the spirit of the present invention and/or the scope of the appended claims.

BASIC PROCEDURES

All peptides were synthesized by Sigma-Genosys, Inc. using conventional techniques. The released peptides were purified to >95% homogeneity via RP-HPLC by the company. The pooled eluted peak material was desalted and lyophilized. Mass Spec analysis confirmed the peptide molecular weight and purity. Unless otherwise noted, all chemicals were purchased from Sigma Chemical Corp. or from Fluka Chemical Co. Active MMP-9 enzyme was purchased from Calbiochem.

Molecular modeling utilized two visualization programs, Swiss PDB Viewer (Guex and Peitsch, 1997) and Rasmol (Sayle and Milner-White, 1995). Model work was performed on a Compaq PC running Windows 95, as well as a Silicon Graphics, Inc. Octane UNIX workstation. Additionally, the Cerius2 molecular package from Molecular Simulations, Inc. was utilized on the Octane. Three dimensional structure files were downloaded from the Protein Databank as follows (filename, reference): MMP-1 (1FBL, Li *et al.*, 1995), MMP-2 (1GEN, Libson *et al.*, 1995), MMP-8 (1JAO, 1JAN, Grams, *et al.*, 1995; Reinemer *et al.*, 1994), MMP-9 (1MMQ, Browner *et al.*, 1995), TIMP-2/MT-1 MMP complex (1BUV, Fernandez-Catalan *et al.*, 1998), TIMP-2 (1BR9, Tuuttila *et al.*, 1998), and TIMP-1/MMP complex (1UEA, Gomis-Ruth *et al.*, 1997; Huang *et al.*, 1996; Becker *et al.*, 1995). These files were used to analyze the three-dimensional structure

of the proteins, and the chemical nature and identification of conserved and variant amino acids in the MMP-TIMP contact interface. This information was utilized to design a minimalist peptide that could be coupled to a zinc chelator that would bind many MMP enzymes in the region of the catalytic zinc.

EXAMPLE 1. IDA Chelating Peptides

Peptide was resuspended in water to a final concentration of 100 mM. The amino form of IDA was dissolved in a small amount of DMSO followed by the slow addition of water until the compound was at a concentration of 150 mM. To the IDA solution was added N-hydroxysuccinimide (NHS), to a final concentration of 175 mM and N-ethyl-N'-(dimethylaminopropyl)-carbodiimide (EDC) to a final concentration of 400 mM. The solution was incubated at 37 °C with gentle stirring for 30 minutes. The previously prepared peptide solution was added to this reaction slowly over a period of 5 minutes. Stirring continued for an additional 30 minutes. The reaction was quenched by the addition of ethanolamine-HCl to a final concentration of 1.0 M. The final mixture was taken to dryness in a rotovac over a period of 10 hours. The solid material was then resuspended in 500 µL of water and the chelating peptide was purified away from unreacted species via RP-HPLC. A 250 mm x 100 mm, 5 ml Hypersil ODS-2 RP column was chromatographed with a mobile phase of: **A**: 0.1% TFA/water, **B**: 0.1%

TFA/acetonitrile. After sample injection, a gradient of 100% **A** (0 to 2 min) and 0-60% **B** (2 to 25 min) was applied. The chelating peptide was detected at 214 nm and was 96% pure by peak integration. The eluting peaks were pooled, mixed with 3 volumes of water, and lyophilized.

- 5 The resulting powder was resuspended in water and dialyzed through a 500 MWCO cellulose acetate dialysis tube versus 50% phosphate buffered saline (PBS)/water. Chelating peptide was aliquoted and stored frozen at -20°C .

10 Example 2. AFTA Chelating Peptides

- AFTA (50 mg) was dissolved in a small amount of DMSO followed by the slow addition of water until the compound was at a concentration of 200 mM. To this solution was added zinc sulfate to a final concentration of 300 mM. This solution was stirred slowly at room
- 15 temperature for 15 minutes. The sample was then treated with EDC/NHS as described in Example 1. The resulting AFTA-succinamide ester was coupled with a peptide having SEQ ID No. 7 or 8, and the resulting peptide was purified as discussed in Example 1. After lyophilization, the AFTA-peptide was resuspended in water, and it was
- 20 extensively dialyzed versus water in a 500 MWCO cellulose acetate dialysis tube. The absence of zinc ion was confirmed by atomic absorption spectroscopy.

Example 3. PSDE Chelating Peptides

PSDE was prepared according to the method of Hayward *et al* (1995). This material was coupled directly to a stoichiometric amount of a cysteine containing peptide having SEQ ID No. 4 or 6 by the following disulfide exchange reaction. PSDE in an amount of 100 mM in water and peptide in an amount of 100 mM in 25 mM Tris-HCl (pH 7.2) were mixed and allowed to react for 45 minutes at 30 °C. The product was purified from the reactants by dialysis versus 10 mM Tris-HCl (pH 7.2) in a 500 MWCO cellulose acetate dialysis tube. The product was further purified by RP-HPLC as described in Example 1.

Example 4. Regulation of MMPs

Two enzymatic assays were performed. The first assay measured the enzymatic hydrolysis of fluoresceinated collagen by MMP-9 as a function of time. Fluoresceinated collagen (Molecular Probes, Inc.), at a concentration of 5 μ M was added to reaction buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM CaCl_2 , 0.1 mM NaN_3) and was placed into a Spectrosil quartz fluorometer cuvette. MMP at a concentration of 0.1 μ M was mixed with varying amounts of chelating peptide having SEQ ID No. 4 or 6 and incubated at 25°C for 10 minutes to effect binding. The protein mixture was added to the collagen substrate and mixed quickly. Fluorescence emission intensity at 520 nm

was measured as a function of time (excitation wavelength 495 nm) in a Shimadzu RF5301 fluorometer. The fluorescein release assay was used to determine the inhibitory constant (K_i) of the chelating peptide inhibitor ([I]) according to Segel (1993) via the use of Dixon plots ($1/v$ vs. [I]), such that:

$$\text{slope} = K_m / (V_{\max} K_i [S]) \quad (1)$$

where K_m is the Michaelis constant, V_{\max} is the reaction maximum velocity, and [S] is the substrate concentration.

The second assay utilized the technique of fluorescence resonance energy transfer (FRET). The substrate peptide (Calbiochem #444221) comprised seven amino acids, coupled to a carboxyl terminal dinitrophenyl acceptor, and an amino terminal 2-aminobenzo-anthraniloyl (Abz) moiety donor. Cleavage of this substrate by MMP-9 results in the liberation of a fluorescent product (365 nm excitation, 450 nm emission). Peptide at a concentration of 5 μ M was added to reaction buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM CaCl_2 , 0.1 mM NaN_3) and placed into a black 96-well microtiter plate well that had been previously blocked with 1% BSA. MMP at a concentration of 0.1 μ M was mixed with varying amounts of chelating peptide (0, 0.01, 0.02, 0.04, and 0.1 μ M) and incubated at 25 $^\circ\text{C}$ for 10 minutes to effect binding. The protein mixture was added to the peptide substrate and mixed quickly. Fluorescence intensity as a function of time was

measured with a Dynex MFX fluorescence microtiter plate reader. Fluorescence intensity was related back to moles of peptide cleaved by producing a standard curve with an Abz containing non-FRET peptide. Inhibitory constants were derived from the curves and are listed below in

5 Table 2.

Table 2. Inhibitor Constants

Peptide	Sequence	Chelator	$K_i(\mu\text{M})$
ChePep-1	CSAVPVH (SEQ ID No. 4)	PSDE	98
ChePep-2	DSAVPVH (SEQ ID No. 5)	IDA	67
ChePep-3	IYTACMSAV (SEQ ID No. 6)	PSDE	350
ChePep-4	IYTACMSAV (SEQ ID No. 6)	IDA	163
ChePep-5	VHTHLCD (SEQ ID No. 7)	AFTA	221
ChePep-6	CTCVP (SEQ ID No. 8)	AFTA	125

Example 5. Surface Plasmon Resonance

10 The BiaCore-X surface plasmon resonance (SPR) device was utilized to measure the interaction between the chelating peptide (CP) and MMP-9. For these experiments a carboxymethyl dextran sensor chip (BiaCore, AB; CM-5, Lofas *et al.*, 1993) was activated with 50 mM N-hydroxysuccinimide, 0.2 M N-ethyl-N'-
 15 (dimethylaminopropyl)-carbodiimide at a flow rate of 10 μL per minute for ten minutes. MMP-9 at a concentration of 75 ng/ μL was coupled to the activated surface at a flow rate of 10 μL per minute for ten minutes. The final surface was inactivated by flowing 1 M ethanolamineHCl at a rate of 10 μL per minute for five minutes over the sensor surface. CP

was flowed over the sensor surface at a rate of 20 μ L per minute, and at concentrations of 10, 25, and 50 nM. Binding isotherms were evaluated by simultaneously fitting the forward (k_a) and reverse (k_d) rate constants to the following formula:

$$5 \quad d[\text{CP} \sim \text{MMP-9}] / dt = (k_a [\text{CP}] [\text{MMP-9}]) - (k_d [\text{CP} \sim \text{MMP-9}]) \quad (2)$$

(Karlsson and Falt, 1997) where [CP], [MMP-9], and [CP~MMP-9] are the concentrations of free chelating peptide, free MMP9, and the complex respectively. The equilibrium affinity constant (K_A) is defined as:

$$10 \quad K_A = k_a / k_d \quad (3)$$

Equation 3 is expressed in terms of the SPR signal (Morton *et al.*, 1995) as:

$$dR/dt = k_a C R_{\max} - (k_a C + k_d) R \quad (4)$$

where R is the SPR signal (in response units, RU) at time t, R_{\max} is the maximum MMP-9 binding capacity in RU, and C is the chelating peptide concentration. Kinetic analysis (O'Shannessy *et al.*, 1993) was performed using Origin from Microcal, Inc.

Example 6. Viability Assays

20 The relative toxicity of the chelating and substrate peptides was assayed using the skin model Epiderm that is commercially

available from MatTek Corp. The individual skin sample containers were preincubated in culture medium at 37 °C under 5% CO₂ for two hours prior to the addition of the peptide constructs. The sample containers were transferred to 6 well plates that contained fresh media.

- 5 All peptides were dissolved in PBS at a final concentration of 10 mM, and 100 µL of each peptide solution was pipetted onto the surface of the Epiderm sample container. Incubation was continued for 12 hours at 37 °C under 5% CO₂. After the incubation period, the sample containers were washed three times with PBS and the sample containers were
- 10 transferred to a 24 well plate that contained 300µL of MTT assay media per well (MTT concentration was 1 mg/mL). The colorimetric assay was allowed to develop for three hours (incubation at 37°C under 5% CO₂). Sample containers were then transferred to a 24 well culture plate that contained 2 mL of isopropanol per well. Extraction of the colored
- 15 precipitate occurred over a period of four hours at room temperature. Absorbance readings were taken at 570 nm and 650 nm for each sample. The percent viability of each sample relative to a PBS control was calculated as:

$$100 \times (\text{OD}_{570}^{\text{sam}} - \text{OD}_{650}^{\text{sam}}) / (\text{OD}_{570}^{\text{con}} - \text{OD}_{650}^{\text{con}}) \quad (5)$$

- 20 Routinely, each peptide sample was assayed in duplicate or triplicate.

Example 7.

A skin equivalent toxicity model (Epiderm) was employed to measure the overall cellular viability in the presence of the six peptide constructs. A single dose of 10 mM peptide (in PBS) was applied to the Epiderm samples for a period of 12 hours. The resulting viability is plotted in Figure 7. A PBS control is set to a value of 100 percent viability. The surfactant Triton X-100 served as a negative control, that is the application of a 1% triton solution should result in over 90% cell death. As can be seen in Figure 7, all six peptides exhibit about 94.2 +/- 3.8 percent. Of the three zinc chelators, AFTA seems to be slightly more toxic (90.5 % viability) than IDA (94.8 % viability) or PSDE (96.5 % viability), although the viability differences are not significant.